

The Autoimmune Blistering Skin Disease Bullous Pemphigoid

The Presence of Plasmin/ α_2 -Antiplasmin Complexes in Skin Blister Fluid Indicates Plasmin Generation in Lesional Skin

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Abstract

Plasminogen activators produced locally in the skin have been implicated in blistering skin diseases. To explore whether plasminogen activators convert their substrate plasminogen into plasmin locally in the lesional skin we have analyzed the autoimmune blistering skin disease bullous pemphigoid. Enzyme activity was detected in bullous pemphigoid skin blister fluid by using a low molecular weight chromogenic substrate for plasmin. Enzyme activity was detected neither in suction blister fluid raised on normal skin nor in normal plasma. Immunoprecipitation or fractionation by molecular sieve chromatography of bullous pemphigoid skin blister fluid followed by testing in immunoassays disclosed putative plasmin/ α_2 -macroglobulin complexes and plasmin/ α_2 -antiplasmin complexes. Enzyme activity detected in bullous pemphigoid skin blister fluid by the low molecular weight chromogenic peptide assay was ascribed to the putative plasmin/ α_2 -macroglobulin complexes. Because formation of plasmin-inhibitor complexes requires the active plasmin, our findings indicate previous activation of plasminogen to plasmin in skin lesions. There was no evidence for free plasmin (i.e., plasmin not complexed to inhibitors) in bullous pemphigoid blister fluid, suction blister fluid, or plasma. (*J. Clin. Invest.* 1993. 92:978-983.) Key words: α_2 -antiplasmin • α_2 -macroglobulin • plasmin-inhibitor complexes • bullous pemphigoid

Introduction

Via activation of plasminogen to plasmin, cellular plasminogen activators are thought to be crucially involved in pericellular proteolysis (1, 2). Plasminogen is a \approx 90-kD single-chain glycoprotein, produced primarily in the liver and present in plasma and the interstitial fluid (3). By immunohistochemistry plasmin(ogen) (This term is used when immunological detection by antibodies, which usually cannot discriminate between plasminogen and plasmin, is meant.) has been localized to the basal layer of the normal human epidermis (4-6). Because there is no evidence for synthesis of plasminogen by keratinocytes in vivo or in vitro it is assumed that basal keratinocytes, in analogy to other cell types (7), bind exogenous plasmin(ogen) from plasma or the interstitial fluid. Binding of plasmin(ogen) to the basal and suprabasal layers of normal

human epidermis involves specialized molecular domains of the plasminogen molecule: the kringle 5AH site and lysine-binding sites of kringles 1-3 (6).

The activity of plasmin is counteracted by its specific inhibitor α_2 -antiplasmin (8) and by the broad-specific proteinase inhibitor α_2 -macroglobulin (9, 10). Both proteinase inhibitors are synthesized in the liver and are present in plasma and the interstitial fluid (9). α_2 -Antiplasmin is a single-chain glycoprotein of 67 kD. It interacts with the active site of plasmin, resulting in covalent binding and formation of a 1:1 stoichiometric, functionally inactive complex (11). α_2 -Macroglobulin is a second-line inhibitor of plasmin, in that it is thought to counteract plasmin when the specific inhibitory potential of α_2 -antiplasmin is exhausted (2, 10). Plasmin complexed to α_2 -macroglobulin retains its enzymatic activity: lower molecular weight (< 20,000) substrates, including low molecular weight chromogenic peptide substrates, can still be cleaved, whereas the degradation of high molecular weight (> 20,000) substrates (including the plasmin substrate fibrin) is sterically prevented (10).

Cellular plasminogen activators convert plasminogen to plasmin, thus, providing the broad-specific proteolytic potential of plasmin for pericellular proteolysis (1, 2, 12, 13). Plasmin generation is favored by localization of the involved reactants to the cell surface (12, 13). When bound to specific cell surface receptors or binding sites, the interaction of plasminogen activators and plasminogen results in the generation of cell surface-bound plasmin (13). Cell surface-bound plasmin is efficiently protected against its specific inhibitor α_2 -antiplasmin (12-14). Thus, cell surfaces are equipped with the proteolytic potential of plasmin. When, however, plasmin is released from its surface binding sites into solution, it can rapidly be inactivated by the specific inhibitor α_2 -antiplasmin (14).

The bullous pemphigoid is an autoimmune skin disease characterized by the formation of subepidermal blisters (15). There is circumstantial evidence for plasminogen activation in lesional skin of the bullous pemphigoid: (a) keratinocytes in lesional skin of bullous pemphigoid express plasminogen activators (16, 17); (b) by immunohistochemistry, a conspicuous deposition of plasmin(ogen) in lesional epidermis of the bullous pemphigoid was observed (18); and (c) "plasmin-like" enzyme activity has been found in the fluid of bullous pemphigoid skin blisters by fibrin zymography (19). The latter activity, however, had not been characterized in detail. The present study was undertaken to explore the molecular nature of the enzyme activity and to identify products that result from the interaction of plasmin with α_2 -antiplasmin and/or α_2 -macroglobulin in the fluid of bullous pemphigoid skin blisters.

Methods

Materials. Plasminogen (No. 41304), and plasmin (15.7 CU/mg; No. 41303) were from Pharmacia/KabiVitrum (Freiburg, Germany).

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High molecular weight (54 kD) uPA (Ukidan) was kindly provided by Serono Laboratories Inc. (Freiburg, Germany). Buffer salts and detergents were of analytical grade from Merck (Darmstadt, Germany). α_2 -Antiplasmin and α_2 -macroglobulin purified from human plasma were a kind gift of Dr. N. Heimburger (Behringwerke, Marburg, Germany). Human standard plasma (No. AO 41115) containing defined amounts of plasminogen, α_2 -antiplasmin, and α_2 -macroglobulin was purchased from Behringwerke.

The chromogenic plasmin substrate CS 2251. The chromogenic peptide substrate H-D-valyl-L-leucyl-L-lysine-L-p-nitroanilide dihydrochloride (S 2251; No. 41206) was purchased from Pharmacia/Kabi-Vitrum. Plasmin splits the substrate S 2251 rapidly. The original specificity screenings of the substrate, which included several other naturally occurring serine proteinases (including thrombin, plasma kallikrein, urokinase- and tissue-type plasminogen activator, hageman factor, coagulation factor Xa, PMN-elastase, and cathepsin G) were summarized by Claeson and co-workers (20, 21) and Friberger (22). The screenings had revealed that there are some similarities between plasmin and kallikrein from pancreas but that plasmin alone prefers COOH-terminal lysine and that L-leucine in position 2 greatly enhanced the substrate properties toward plasmin. The preference of plasmin for COOH-terminal lysine is also the case with its natural substrate fibrin, where 20 out of 32 bonds cleaved are lysine (22). Because of its specificity for plasmin the substrate S 2251 is recommended for the determination of plasmin even in the presence of complex matrices, such as plasma (22).

Skin blister fluid. Skin blister fluid was aspirated from bullous pemphigoid blisters generated within 1–2 d. The diagnosis of bullous pemphigoid was made on the basis of clinical, histopathological, and immunopathological findings. The fluid of suction blisters induced on clinically normal skin of volunteers according to the method of Volden (23) was used as control. The suction blisters were allowed to sit for 30 min before aspiration of their contents. In addition, commercially available human standard plasma (see above) was used as control.

Chromogenic substrate assay and fibrinolysis in gel assay. The chromogenic substrate assay buffer was 30 mM Tris/HCl, pH 7.4, 60 mM NaCl, 0.05% Na₂S₂O₅, 0.1% Triton X-100 (assay buffer/Triton). 60 μ l of the respective sample, diluted appropriately in assay buffer/Triton, was admixed to 20 μ l assay buffer/Triton and 20 μ l of the chromogenic substrate (stock solution 2 mg/ml in assay buffer without Triton). The assay was performed in flat-bottom micro-titer plates and the absorbance change at 405 nm, caused by the liberation of nitroaniline, was followed by using an automated microplate reading apparatus.

Fibrin agar gels for the detection of plasmin activity were prepared as described previously (24); the gels did not contain plasminogen. Wells of 7-mm diam were punched out and filled with 20 μ l of plasmin, standard plasma, or blister fluid diluted in PBS. Diffusion of proteins into the gel followed by radial fibrinolysis was allowed to proceed for 48 h at 37°C. Subsequently, the plate was fixed with methanol/acetic acid/water (33:7:60), stained with 0.1% (wt/vol) Coomassie brilliant blue R-250 in methanol/acetic acid/water (40:10:50), and finally destained with the solution used for fixation. The lower limit for the detection of plasmin activity in the fibrinolysis assay was 0.1 U of plasmin/ml.

ELISAs. ELISAs for the detection and quantification of α_2 -macroglobulin were carried out as previously described in detail (25). The assays for determination of plasmin(ogen), α_2 -antiplasmin, and plasmin/ α_2 -antiplasmin complexes were similarly performed, except that the following antibody preparations and dilutions were used as solid phase-bound or detecting antibody: (a) for detection of plasmin(ogen): solid phase-bound antibody = polyclonal rabbit anti-human plasminogen IgG (No. B 104808; Behringwerke) at 0.2 μ g/ml; detecting antibody: a mixture of murine monoclonal anti-plasmin(ogen) antibodies HD-PG 2 and HD-PG 7 (18) at 0.2 μ g/ml. The assay detects plasmin in plasmin/ α_2 -antiplasmin complexes but not in plasmin/ α_2 -macroglobulin complexes. (b) for detection of α_2 -antiplasmin: solid phase-bound antibody = polyclonal anti- α_2 -antiplasmin IgG (AHP-07; Serotec via Camon Laborservice; Wiesbaden, Ger-

many) at 0.2 μ g/ml; detecting antibody = murine monoclonal anti- α_2 -antiplasmin antibody HD-AP 1 at 2 μ g/ml. (c) for detection of plasmin/ α_2 -antiplasmin complexes: solid phase-bound antibody = polyclonal anti-plasminogen IgG at 0.2 μ g/ml; detecting antibody = murine monoclonal anti- α_2 -antiplasmin antibody HD-AP 1 at 2 μ g/ml. Bound "detecting" antibodies were quantified by addition of a peroxidase-labeled goat anti-mouse IgG antibody preparation and orthophenyldiamin as substrate (25).

Graded amounts of purified antigen and human standard plasma with a known concentration of the respective antigens were taken for calibration. Fractions after molecular sieve chromatography were tested at a final dilution of 1:15 in PBS/0.05% Tween 20. A standard preparation of plasmin/ α_2 -antiplasmin complexes was prepared by mixing plasmin and α_2 -antiplasmin at a 1:1 (wt/wt) ratio and incubation for 15 min at room temperature.

Molecular sieve chromatography. Molecular sieve chromatography was performed by using a fast protein liquid chromatography apparatus equipped with a superose 6 column (model 17-0537-01; Pharmacia). 50 μ l of the respective sample (a 1:5 dilution of standard plasma in PBS or undiluted skin blister fluids) was applied and eluted at a flow rate of 0.5 ml/min. Fractions of 0.5 ml vol were collected. Elution buffer was: 50 mM sodium phosphate, 150 mM NaCl, pH 7.4.

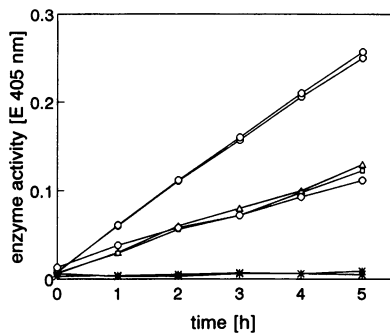
Immunoprecipitation. Either BSA, monoclonal antiplasmin(ogen) antibody (clone HD-PG 19, which does not recognize plasmin bound to α_2 -macroglobulin; [18]), or monoclonal anti- α_2 -macroglobulin antibody (clone HD-a2M 2; [25]) were immobilized to activated CH-Sepharose 4B (No. 17-0490-01; Pharmacia) according to the manufacturer's protocol. 100 μ l of the BSA- or antibody-Sepharose conjugate was added to 100 μ l of a 1:20 dilution of bullous pemphigoid skin blister fluid in assay buffer/Triton. The blister fluid used for immunoprecipitation was a mixture of fluids derived from six different blisters of two different patients. The mixture of sepharose and blister fluid was rotated end-over-end for 2 h at room temperature. Afterwards, sepharose (pellet) and soluble material (supernatant) were separated by centrifugation at 10,000 g for 2 min. 100 μ l of assay buffer/Triton was added to both preparations followed by chromogenic substrate S 2251. The enzyme reaction was allowed to proceed for 7 h at 37°C. Afterward both preparations were spun again at 10,000 g for 10 min, 100 μ l of the solution was pipetted into a flat-bottom microtiter plate, and the absorbance at 405 nm was determined.

Results

Analysis of unfractionated skin blister fluids. Fluid was aspirated from skin blisters of bullous pemphigoid patients or from suction blisters induced on clinically normal skin of volunteers. The blister fluids were tested in a chromogenic assay using the plasmin substrate S 2251. Enzyme activity was detectable in bullous pemphigoid but not in suction blister fluid (Fig. 1). Activity in bullous pemphigoid skin blisters corresponded to 1.2 U of plasmin/ml. When, however, blister fluids were tested in a radial fibrinolysis assay, no fibrinolytic activity was detectable (data not shown), although fibrin zymography (detection limit 0.1 U of plasmin/ml) should have allowed detection of the respective amount of plasmin.

Analysis of fractionated skin blister fluids. Skin blister fluids or human standard plasma were fractionated by molecular sieve chromatography (Superose 6). In fractions of bullous pemphigoid skin blister fluids two peaks of enzyme activity cleaving the plasmin substrate S 2251 were observed: a high molecular weight peak corresponding to a molecular mass of ~ 700–900 kD (elution volume ¹ [K_{av}] 2–4 ml) and a lower molecular weight peak corresponding to ~ 90–100 kD (K_{av}

1. Abbreviation used in this paper: K_{av} , elution volume.



chromogenic substrate assay. After different intervals of time (abscissa) the absorbance at 405 nm (ordinate) was determined. Enzyme activity was observed in bullous pemphigoid blister fluids but not suction blister fluids.

6.5–9 ml; Fig. 2 A, circles). In fractions of suction blister fluids (Fig. 2 A, squares) or human standard plasma (Fig. 2 B, asterisks) only the low molecular weight peak of enzyme activity was observed. The low molecular weight peak is likely to represent free plasmin, because (a) the peak comigrates with the activity peak obtained after fractionation of purified plasmin (Fig. 2 B, triangles), (b) plasmin(ogen)-specific immunoreactivity is detected in fractions containing the low molecular weight plasmin activity (Fig. 3 A; note that plasmin(ogen) is present in fractions of bullous pemphigoid and suction blisters), (c) upon prolonged incubation (48–96 h) the activity could be detected in the fibrinolysis assay (data not shown), (d) enzyme activity in these fractions is inhibited by the plasmin inhibitor aprotinin (data not shown), and (e) enzyme activity in these fractions is increased by pretreatment with exogenous urokinase-type plasminogen activator (Fig. 3 B). The latter finding indicates the additional presence of plasminogen, which has the same molecular weight as plasmin, in the respective fractions.

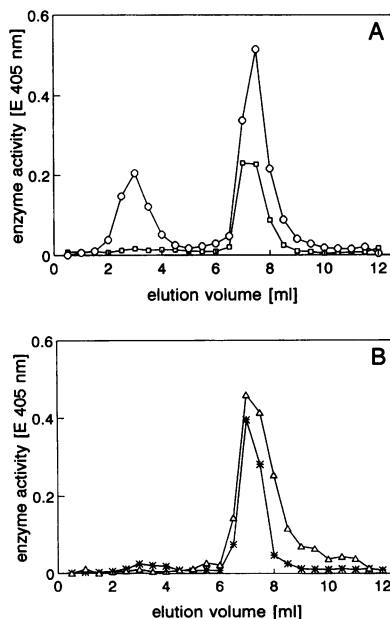


Figure 2. S 2251-cleaving enzyme activity in fractionated bullous pemphigoid skin blister fluid (A, circles), suction blister fluid (A, squares), human standard plasma (B, asterisks), or plasmin (B, triangle). 50 μ l of the respective sample were separated by Superose 6 chromatography as described in Methods. The enzyme activity in individual fractions was determined by using the chromogenic substrate S 2251. A representative experiment is depicted: eight distinct blister fluids of four bullous pemphigoid patients were analyzed and displayed a comparable elution profile. The enzyme reaction was allowed to proceed for 24 h.

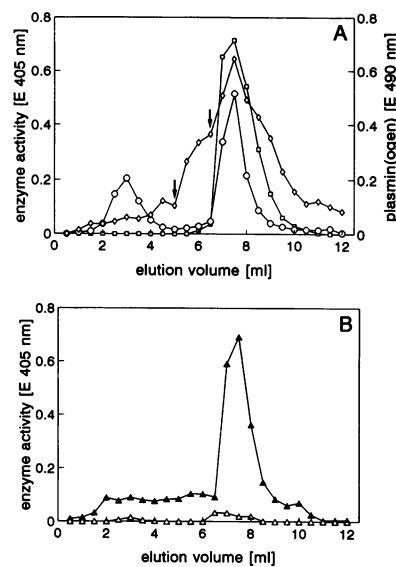


Figure 3. Plasmin(ogen) in fractionated bullous pemphigoid or suction skin blister fluid. Skin blister fluids were separated by Superose 6 chromatography as described in Methods. (A) Individual fractions were tested in a plasmin(ogen)-specific ELISA. The immunoassay revealed plasmin(ogen) in the lower molecular weight plasmin activity-containing fractions (K_{av} 6.5–9.0 ml) in skin blister fluid of bullous pemphigoid (diamonds) or suction (squares) blisters. For

comparison the plasmin activity in the individual fractions of separated bullous pemphigoid blister fluid is shown (circles). Arrows, a shoulder of plasmin(ogen)-specific reactivity according to K_{av} 5.0–6.5 ml was observed in the fractions of bullous pemphigoid blister fluid. (B) Individual fractions of a separated bullous pemphigoid blister fluid were preincubated with uPA (400 IE/ml, 30 min, room temperature). Afterwards the chromogenic plasmin substrate S 2251 was added to pretreated (filled triangles) and untreated (open triangles) samples. Note that the enzyme reaction was allowed to proceed for only 4 h at 37°C. Enhancement of plasmin activity by uPA-pretreatment was observed in the lower molecular weight plasmin activity-containing fractions (K_{av} 6.5–9.0 ml). The finding indicated the presence of plasminogen, which has the same molecular weight as plasmin, in these fractions. Similar findings were obtained in fractions of suction blister fluid (not shown).

α_2 -Antiplasmin/plasmin complexes in bullous pemphigoid skin blister fluid. The analysis of fractionated bullous pemphigoid blister fluid for plasmin(ogen) by ELISA revealed a broad peak of reactivity (Fig. 3 A): activity ranged from the fractions that contained enzyme activity (K_{av} 6.5–9 ml) to higher molecular weight fractions (K_{av} 5.0–6.5 ml). Because the ELISA for plasmin(ogen) cannot discriminate between free plasmin(ogen) and plasmin/ α_2 -antiplasmin complexes, the shoulder of immunoreactivity in the higher molecular weight region (indicated by arrows in Fig. 3 A; K_{av} 5.0–6.5 ml) suggested the presence of plasmin/ α_2 -antiplasmin complexes, the molecular mass of which is \approx 70 kD higher (\approx 160 kD) than that of free plasmin or plasminogen (both having a molecular mass of \approx 90 kD). The presence of such complexes was tested in an ELISA that is specific for plasmin/ α_2 -antiplasmin complexes (see Methods). Plasmin/ α_2 -antiplasmin complexes (K_{av} 5.0–7.0 ml) that comigrated with artificially prepared complexes (Fig. 4 B, triangles) were detected in fractions of bullous pemphigoid skin blister fluid (Fig. 4 A, circles) but not in fractions of suction blister fluid (Fig. 4 A, squares) or of human standard plasma (Fig. 4 B, asterisks).

Putative plasmin/ α_2 -macroglobulin complexes in bullous pemphigoid skin blister fluid. When fractions of bullous pemphigoid skin blister fluid were tested in an α_2 -macroglobulin-specific ELISA, the high molecular weight peak of enzyme activity (Fig. 5, circles; K_{av} 2–4 ml) comigrated with α_2 -macro-

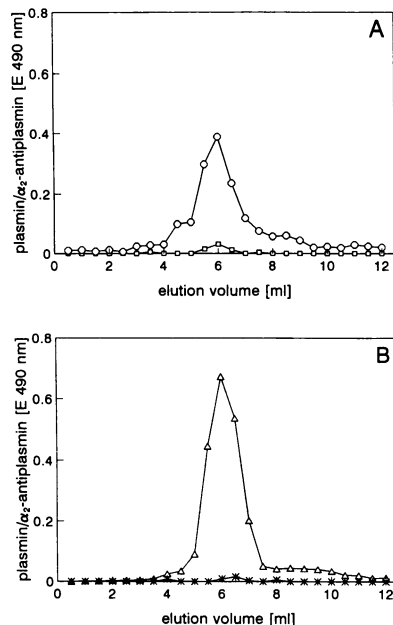


Figure 4. Determination of plasmin/ α_2 -antiplasmin complexes. 50 μ l of bullous pemphigoid skin blister fluid (A, circles), suction blister fluid (A, squares), human standard plasma (diluted 1:5 in PBS; B, asterisks) or artificially prepared plasmin/ α_2 -antiplasmin complexes (B, triangles) was separated by Superose 6 chromatography as described in Methods. The individual fractions were tested in the ELISA specific for plasmin/ α_2 -antiplasmin complexes. Reactivity was observed in fractions (K_{av} 5.0–7.5 ml) of bullous pemphigoid skin blister fluid and of artificially prepared plasmin/ α_2 -antiplasmin complexes.

globulin (Fig. 5, squares). Additional evidence for the presence of putative plasmin/ α_2 -macroglobulin complexes was derived from immunoprecipitation experiments. The S 2251-cleaving enzyme activity present in bullous pemphigoid skin blister fluid could efficiently be precipitated by immobilized anti- α_2 -macroglobulin antibodies (Table I).

No enzyme activity was observed in the high molecular weight region (K_{av} 2–4 ml) when plasma or suction blister fluid were separated (Fig. 2). Quantification by ELISAs revealed that the amounts of total α_2 -macroglobulin were in the same range in bullous pemphigoid and suction blister fluid and that K_{av} of α_2 -macroglobulin in these specimens was identical to that of purified α_2 -macroglobulin (not shown).

Discussion

Enzyme activity, which cleaves the chromogenic plasmin substrate HD-Val-Leu-Lys-pNA (S 2251), was detected in blister fluid of bullous pemphigoid skin lesions. Molecular sieve chromatography of the blister fluid revealed a high molecular

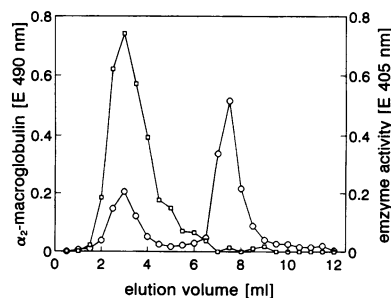


Figure 5. α_2 -Macroglobulin in fractionated bullous pemphigoid skin blister fluid. Bullous pemphigoid skin blister fluid was separated by Superose 6 chromatography as described in Methods. α_2 -Macroglobulin in individual fractions was determined by using an

α_2 -macroglobulin-specific ELISA (squares). For comparison S 2251-cleaving enzyme activity in the respective fractions is shown (circles). High molecular weight enzyme activity coeluted with α_2 -macroglobulin (K_{av} 2–4 ml).

Table I. Immunoprecipitation of S 2251-cleaving Enzyme Activity in Bullous Pemphigoid Skin Blister Fluid

Precipitation with	Enzyme activity precipitated A_{405}
BSA-Sephacrose	0.045
Anti-plasmin(ogen)-Sephacrose (HD-PG 19)	0.066
Anti- α_2 -macroglobulin-Sephacrose (HD-a2M 2)	0.202

A mixture of six different blister fluids derived from two different bullous pemphigoid patients was used for immunoprecipitation as described under Methods. Sepharose was conjugated with BSA, anti-plasmin(ogen) mAbs derived from hybridoma clone HD-PG 19 (31), or anti- α_2 -macroglobulin mAbs derived from hybridoma clone HD-a2M 2 (25). Precipitated enzyme activity was determined by using the chromogenic plasmin substrate S 2251. The enzyme reaction was allowed to proceed for 7 h at 37°C. Data is given as absorbance at 405 nm. It appears that S 2251-cleaving activity is precipitated by anti- α_2 -macroglobulin-Sephacrose beads.

weight peak of activity (corresponding to putative plasmin/ α_2 -macroglobulin complexes), a low molecular weight peak of activity (corresponding to free plasmin), and functionally inactive plasmin/ α_2 -antiplasmin complexes. No enzyme activity was detected in nonfractionated suction blister fluid or in plasma. Our data, in particular the demonstration of plasmin/ α_2 -antiplasmin complexes the formation of which requires the interaction between the inhibitor and the active plasmin, are evidence for the generation of plasmin at some stage of lesion formation in the bullous pemphigoid.

Proteolytic enzymes, including plasmin, bound by α_2 -macroglobulin retain their enzymatic activity; because of steric hindrance they are only able to cleave substrates with lower molecular weight (< 20,000) (10). Thus, the protease activity in nonfractionated bullous pemphigoid skin blister fluid, which was detected by the chromogenic peptide assay (compare Fig. 1; molecular mass of the substrate \approx 500) and which could be precipitated by immobilized by anti- α_2 -macroglobulin antibodies (Table I), is likely to be due to protease (plasmin?)/ α_2 -macroglobulin complexes (compare Table I and Fig. 5). This notion is further substantiated by the finding that enzyme activity was not detected by using the fibrin gel zymography; although the relative sensitivity of the fibrin gel zymography should have allowed detection of plasmin activity.

The specificity of the substrate S 2251 for plasmin was indicated by the fact that it cannot significantly be cleaved by other naturally occurring trypsin-like enzymes, such as thrombin, plasma kallikrein, uPA, tPA, hageman factor, coagulation factor Xa, PMN-elastase, or cathepsin G (22). These substrate properties were taken to conclude that it is plasmin that is responsible for the turnover of the substrate S-2251 in bullous pemphigoid skin blister fluid (Fig. 1). However, we cannot exclude that the turnover of the substrate is due to other, possibly as yet undefined, S 2251-cleaving enzymes. Therefore, the α_2 -macroglobulin complexes containing S 2251-cleaving activity are denominated as putative plasmin/ α_2 -macroglobulin complexes.

On the other hand, plasmin and α_2 -antiplasmin form a stable complex that is functionally inactive (8). The complex could be detected by using an ELISA based on two antibodies with different specificities: one antibody specific for plas-

min(ogen) and one with specificity for α_2 -antiplasmin. The assay revealed the presence of plasmin/ α_2 -antiplasmin complexes in bullous pemphigoid blister fluid but not in suction blister fluid or in plasma. Because the formation of plasmin/ α_2 -antiplasmin complexes requires the interaction of active plasmin with α_2 -antiplasmin the finding provided strong evidence for the presence of plasmin at some stage in lesional skin of the bullous pemphigoid.

By casein zymography Lauharanta and co-workers (19) demonstrated plasmin-like enzyme activity in 3 of 10 bullous pemphigoid skin blister fluids. In zymography the enzyme activity comigrated with plasmin (molecular mass \approx 90 kD, thus denominated plasmin-like) in both bullous pemphigoid and suction blister fluid; several high molecular weight plasmin complexes, however, were solely observed in bullous pemphigoid blister fluid. We did not find evidence for a 90-kD plasmin-like activity in fibrin zymography (not shown). So far, we can only speculate on the observed variance. One explanation could be that in the study of Lauharanta et al. (19) either during preparation of the blister samples or during zymographic analysis plasminogen has been converted to plasmin, which then accounts for the enzyme activity migrating at 90 kD. That generation of plasmin could be an artifact of sample preparation is already indicated by our findings that plasmin generation is an apparent artifact caused by molecular sieve fractionation of blister fluids or plasma (see also next paragraph of the Discussion). Besides that, it remains open, whether the high molecular weight complexes observed in the previous study are related to plasmin/ α_2 -antiplasmin or putative plasmin/ α_2 -macroglobulin complexes identified by us.

We did not find enzyme activity in nonfractionated suction blister fluid (compare Fig. 1) or plasma (data not shown). In contrast, after molecular sieve chromatography enzyme activity (Fig. 2; low molecular weight peak of activity) was observed in all specimens. Enzyme activity in these fractions comigrated with genuine plasmin (Fig. 2 B) and was inhibitable by aprotinin (not shown). We thus conclude that we deal with free plasmin in these fractions. It is, however, reasonable to assume that generation of free plasmin occurred secondarily during molecular sieving: separation of plasminogen from plasmin inhibitors during fractionation and subsequent plasminogen activation by trace amounts of plasminogen activators coeluting in plasminogen-containing fractions may account for this artifact. Taken together, we favor the explanation that free plasmin, capable of degrading high molecular weight extracellular protein substrates, such as fibrin, is neither present in skin blister fluids nor in plasma.

Soluble plasmin appearing in plasma or the interstitial fluid is readily counteracted by α_2 -antiplasmin and -macroglobulin (8–10). Cell surface-bound plasmin, in contrast, is protected against inhibition (7, 12, 13). Plasmin generation at cellular surfaces in lesional epidermis of bullous pemphigoid appears likely, because keratinocytes of lesional skin express plasminogen activators (16, 17), and plasmin(ogen) is associated with the epidermis of lesional skin (18). Moreover, cell surface receptors for uPA (26, 27) or plasmin(ogen) (reference 6 and our own unpublished observations) could be demonstrated in human keratinocytes in vitro and in vivo. Plasmin/inhibitor complexes in bullous pemphigoid lesions may thus be formed when plasmin is released from putative epidermal binding sites into the blister cavity, where it encounters α_2 -antiplasmin or -macroglobulin.

Taken together, our data provide evidence that in lesional skin of the bullous pemphigoid mechanisms are operative that lead to the generation of active plasmin. Whether plasmin generation and subepidermal blister formation are causally related or not cannot be decided at present. Although several lines of evidence point at a causal link between plasminogen activation and blister formation in other bullous skin diseases, such as, e.g., the pemphigus vulgaris (28–30), the expression of plasminogen activators has also been observed in the epidermis of nonblistering skin diseases, such as psoriasis (16). In view of these findings, it remains to be explored in more detail whether plasmin generation is involved at some stage in the skin blister formation of bullous dermatoses, including subepidermal blister formation of the bullous pemphigoid.

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References

1. Dano, K., P. A. Andreasen, J. Grondahl Hansen, P. Kristensen, L. S. Nielsen, and L. Skriver. 1985. Plasminogen activators, tissue degradation, and cancer. *Adv. Cancer Res.* 44:139–266.
2. Saksela, O. 1985. Plasminogen activation and regulation of pericellular proteolysis. *Biochim. Biophys. Acta.* 823:35–65.
3. Raum, D., R. Levey, P. O. Taylor, and T. E. Starzl. 1980. Synthesis of human plasminogen by the liver. *Science (Wash. DC)*. 208:1036–1037.
4. Isseroff, R. R., and D. B. Rifkin. 1983. Plasminogen is present in the basal layer of the epidermis. *J. Invest. Dermatol.* 80:297–299.
5. Justus, C., S. Müller, and M. D. Kramer. 1987. A monoclonal antibody recognizing plasminogen and plasmin-altered reactivity in psoriatic lesions. *Br. J. Dermatol.* 117:687–694.
6. Burge, S. M., J. M. Marshall, and S. A. Cederholm-Williams. 1992. Plasminogen binding sites in normal human skin. *Br. J. Dermatol.* 126:35–41.
7. Miles, L. A., and E. F. Plow. 1989. Plasminogen receptors: ubiquitous sites for cellular regulation of fibrinolysis. *Fibrinolysis.* 2:61–71.
8. Collen, D. 1980. On the regulation and control of fibrinolysis. *Thromb. Haemostasis.* 42:77–89.
9. Travis, J., and G. S. Salvesen. 1983. Human plasma proteinase inhibitors. *Annu. Rev. Biochem.* 52:655–709.
10. Van Leuven, F. 1982. Human α_2 -macroglobulin: structure and function. *Trends Biochem. Sci.* 185–187.
11. Wiman, B., and D. Collen. 1979. On the mechanism of the reaction between human α_2 -antiplasmin and plasmin. *J. Biol. Chem.* 254:9291–9297.
12. Pollanen, J., R. Stephens, E. M. Salonen, and A. Vaheri. 1988. Proteolytic mechanisms operating at the surface of invasive cells. *Adv. Exp. Med. Biol.* 233:187–199.
13. Stephens, R. W., J. Pöllänen, H. Tapiovaara, K.-C. Leung, P.-S. Sim, E.-M. Salonen, E. Ronne, N. Behrendt, K. Danö, and A. Vaheri. 1989. Activation of pro-urokinase and plasminogen on human sarcoma cells. A proteolytic system with surface-bound reactants. *J. Cell Biol.* 108:1987–1995.
14. Plow, E. F., D. E. Freaney, J. Plescia, and L. A. Miles. 1986. The plasminogen system and cell surfaces. Evidence for plasminogen and urokinase receptors on the same cell type. *J. Cell Biol.* 103:2411–2420.
15. Farmer, E. R. 1985. Subepidermal bullous diseases. *J. Cutaneous Pathol.* 12:316–321.
16. Jensen, P. J., J. Baird, S. Morioka, S. Lessin, and G. S. Lazarus. 1988. Epidermal plasminogen activator is abnormal in cutaneous lesions. *J. Invest. Dermatol.* 90:777–782.
17. Baird, J., G. S. Lazarus, D. Belin, J. D. Vassalli, N. Busso, P. Gubler, and P. J. Jensen. 1990. mRNA for tissue-type plasminogen activator is present in lesional epidermis from patients with psoriasis, pemphigus, or bullous pemphigoid, but is not detected in normal epidermis. *J. Invest. Dermatol.* 95:548–552.
18. Gissler, H. M., M. M. Simon, and M. D. Kramer. 1992. Enhanced associa-

- tion of plasminogen/plasmin with lesional epidermis of bullous pemphigoid. *Br. J. Dermatol.* 127:272-277.
19. Lauharanta, J., E.-M. Salonen, and A. Vaheiri. 1989. Plasmin-like proteinase associated with high molecular weight complexes in blister fluid of bullous pemphigoid. *Acta Dermato-Venereol.* 69:527-529.
 20. Claeson, G., L. Aurell, G. Karlsson, and P. Friberger. 1977. Substrate structure and activity relationships. In *New Methods for the Analysis of Coagulation Using Chromogenic Substrates*. I. Witt, editor. Walter de Gruyter, Berlin, Germany. 37-48.
 21. Claeson, G., L. Aurell, G. Karlsson, and P. Friberger. 1978. Substrate structure and activity relationships. In *Progress in Chemical Fibrinolysis and Thrombolysis*. J. F. Davidson, R. M. Rowan, M. M. Samama, and P. C. Desnoyers, editors. Raven Press, New York. 299-304.
 22. Friberger, P. 1982. Chromogenic peptide substrates. Their use for the assay of factors in the fibrinolytic and the plasma kallikrein-kinin systems. *Scand. J. Clin. Lab. Invest. Suppl.* 42:1-98.
 23. Volden, G. 1978. Acid hydrolases in blister fluid 3. Characterization and quantification of peptide hydrolases. *Br. J. Dermatol.* 99:49-52.
 24. Brunner, G., and V. Schirmacher. 1988. Fibrin autography of plasminogen activator by electrophoretic transfer into fibrin agar gels. *Anal. Biochem.* 168:411-416.
 25. Justus, C., S. Müller, and M. D. Kramer. 1988. Application of novel monoclonal antibodies in the purification, quantification, and immunohistological localization of the proteinase inhibitor α_2 -macroglobulin. *Enzyme Microb. Technol.* 10:524-531.
 26. Del Rosso, M., G. Fibbi, G. Dini, C. Grappone, M. Pucci, R. Caldini, L. Magnelli, M. Fimiani, T. Lotti, and E. Panconesi. 1990. Role of specific membrane receptors in urokinase-dependent migration of human keratinocytes. *J. Invest. Dermatol.* 94:310-316.
 27. McNeill, H., and P. J. Jensen. 1990. A high-affinity receptor for urokinase plasminogen activator on human keratinocytes: characterization and potential modulation during migration. *Cell Regul.* 1:843-852.
 28. Singer, K. H., N. J. Sawka, H. R. Samowitz, and G. S. Lazarus. 1980. Proteinase activation: a mechanism for cellular dyshesion in pemphigus. *J. Invest. Dermatol.* 74:363-367.
 29. Spillman, D. H., P. H. Magnin, L. Roquel, and M. Mitsui. 1988. Aprotinin inhibition of experimental pemphigus in BALB/c mice following passive transfer of pemphigus foliaceus serum. *Clin. Exp. Dermatol.* 13:321-327.
 30. Takamori, K., T. Yoshiike, S. Morioka, and H. Ogawa. 1988. The role of proteases in the pathogenesis of bullous dermatoses. *Int. J. Dermatol.* 27:533-539.
 31. Meissauer, A., M. D. Kramer, V. Schirmacher, and G. Brunner. 1992. Generation of cell surface-bound plasmin by surface-bound urokinase- or tissue-type plasminogen activator: a key event during melanoma cell invasiveness in vitro. *Exp. Cell Res.* 199:179-190.